# *The role of birds in disseminating ticks and tick-borne diseases of veterinary and medical importance*

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## Abstract

Due to bird migration ticks can be disseminated over long distances and can even cross natural barriers. During migration birds can carry ticks and disseminate them and the pathogens that they may carry from one location to the other. Ticks were collected from birds at two sites in the Netherlands. The ticks sampled came from 15 different bird species. The predominant tick species found was *Ixodes ricinus*, furthermore one female *Hyalomma scupense* was found. Only adults and nymphs were screened for pathogens using PCR and RLB. Results showed that a broad array of pathogens were found in the sampled ticks. With the finding of the female *Hyalomma scupense* that is only present in southern Europe it can be concluded that birds have a role in the dissemination of ticks and tick-borne diseases.

# Introduction

## Bird migration

Bird migration is the annually recurring phenomenon of birds flying to their wintering grounds in the fall to their breeding grounds in the spring[1–4]. Migration eventually is about changing feeding conditions and optimizing them[5]. In the evolution of birds migration developed at an early stage. This means that it has existed for a long time. Studies involving bird migration however, are challenging because of the (small) size of some birds[4–6].

There are different kinds of migrants that can migrate over different distances. Sometimes breeding and wintering grounds can even overlap while other migrants travel from continent to continent. Obligate migrants migrate every year. There is also something known as obligate and facultative partial migration. This means that in a population certain individuals migrate every year while other individuals stay behind or migrate at irregular intervals respectively. Migration is under endogenous control. Birds have a so-called circannual rhythm, a kind of biological clock.

Experiments conducted showed that migratory activity and even migratory behaviour is inheritable. Migratory direction also is genetically transferred, along with the duration and termination of migration[5].

The method mostly used for tracking birds is ringing, with the use of metal rings. It can be used on almost all bird species and if the individual is found the ring offers the finder information about the bird[6]. The information can aid in the interpretation of migration patterns of an individual[4]. Recoveries of ringed individuals made over the years made it clear that breeding and wintering grounds usually are species- even population-specific. However, when environmental conditions change it is of major importance that migratory birds adapt to these new conditions. This has been observed in some studies [5].

Because birds are able to fly they can cross natural barriers such as mountains, oceans and deserts, this means that birds can carry parasites to new areas[1–3,7–11].

## Ticks

Migratory birds are able to fly over long distances this makes it possible that parasites infesting them can be transported with them[1–3,7–11]. One such parasite is the tick. The tick has three stages, larva, nymph and adult. Adult ticks lay eggs, the larva from these eggs feed once before molting into a nymph, the nymph also feeds once before molting into an adult. Only when they have molted into the adult stage they can reproduce. In every stage a tick feeds on blood from a host.

The commonest type of life cycle is the three-host life cycle. This means that there are different hosts for every stage. Suitable hosts and environment must be present for a tick to survive[1,7,9,10,12–14]. Ticks can be reservoir hosts and vectors for several animal diseases and zoonoses[1,3,8–10].

## Birds in this survey

The ticks sampled in this survey came from 15 different bird species. Not all bird species migrate over long distances, breeding and wintering grounds can (somewhat) overlap [6]. These bird species are called medium or short distance migrants [6,15].

The long-distance migrants: Blue throat (*Luscinia svecica);* Common whitethroat (*Sylvia communis*); Eurasian blackcap (*Sylvia atricapilla*)[6,15–17]. Migration directions range from south-east to north-west and from south to north[17]. Long-distance migrants arrive from their wintering grounds from the south-east and the south. They winter in tropical areas such as Africa or even Asia and migrate through central or east-Europe to reach their breeding grounds. The migration period is from April to June [6,15,17,18].

| ***Bird species*** | ***Migration direction*** |
|---|---|
| *Long-distance migrants* | |
| Blue throat (*Luscinia svecica*) | SE-NW |
| Common whitethroat (*Sylvia communis*) | S-N |
| Eurasian blackcap (*Sylvia atricapilla*) | SE-NW |
| *Short/medium-distance migrants* | |
| Common blackbird (*Turdus merula*) | SW-NE |
| Great tit (*Parus major*) | SW-NE |
| Eurasian blue tit (*Cyanistes caeruleus*) | SW-NE |
| Treecreeper (*Certhia familiaris*) | SW-NE |
| European greenfinch (*Chloris chloris*) | SW-NE |
| Chaffinch (*Fringilla coelebs*) | SW-NE |
| Redwing (*Turdus iliacus*) | SW-NE |
| Song thrush (*Turdus philomelos*) | SW-NE |

Table 1: Bird species found in this survey and their migration direction

The short/medium-distance migrants: Common blackbird (*Turdus merula*); Great tit (*Parus major);* Eurasian bleu tit (*Cyanistes caeruleus*); Treecreeper (*Certhia familiaris*); European greenfinch (*Chloris chloris*); Chaffinch (*Fringilla coelebs*); Redwing (*Turdus iliacus*); Song thrush (*Turdus philomelos*); European robin (*Erithacus rubecula*); Dunnock (*Prunella modularis*); Eurasian wren (*Troglodytes troglodytes*); Hawfinch (*Coccothraustes coccothraustes*)[6,15–17]. The short/medium-distance migrants (almost) all migrate from the South-west to the North-East[15,17,18]. Their wintering grounds mainly lay in western and southern Europe and around the Mediterranean. In the spring they migrate to the north-east. They mainly migrate in april[6,15,17].

## Ticks on birds

Studies showed that in Europe blackbirds, song thrushes, European robins, Eurasian wren, Dunnocks and Chaffinches are the most seen tick infested bird species[7,9,10,19]. Of these Blackbirds appear to have the highest prevalence of tick infestation[7,10,12,20]. Song thrushes and European robins also had a high number of tick infestation. Other bird species had a moderate to low prevalence of tick infestation[9,19,20]. Dunnocks and birds of the thrush family feed on the ground, this can be the reason of the high infestation rate [9].

## Tick species in Europe

Tick species found on birds in Europe include *Ixodes ricinus, Ixodes persulcatus, Ixodes frontalis, Ixodes hexagonus, Rhipicephalus sanguineus, Dermacentor reticulatus* and *Haemaphysalis* spp[1–3,7,9–11,13,21,22]. Of these tick species *I. ricinus* was the most abundant tick species found in Europe[9,13,14,21–24]. Indigenous to the Netherlands are *I. ricinus, I. hexagonus* and *Haemaphysalis punctata*[21]. The ticks described are all of the family Ixodidae, they are also called hard ticks, due to the (hard) plates on their bodies [12].

## Pathogens carried by ticks

Ticks can carry bacterial pathogens such as *Anaplasma* spp*,* in particular *Anaplasma centrale, Anaplasma marginale, Anaplasma ovis, Anaplasma phagocytophilum, Borrelia* spp*, Borrelia afzelii Borrelia burgdorferi, , Borrelia garinii, Borrelia valaisiana, Rickettsia* spp*, Rickettsia burnetii (or Coxiella burnetii), Rickettsia rickettsia*[9,22]. Several other protozoan pathogens can also be found such as *Babesia* spp, *Babesia canis, Babesia divergens, Babesia ovis* and *Babesia venatorum*[2,3,7,10,14,22,23]. Furthermore viral pathogens such as Tick-borne encephalitis virus and Crimean-Congo haemorrhagic fever virus can be present in ticks[1,7,9–11,13,19,21,23].

## Diseases

The above mentioned pathogens can be of both veterinary and medical importance. These pathogens cause diseases like anaplasmosis, Lyme borreliosis, ehrlichiosis, babesiosis and rickettsiosis[21].

Lyme borreliosis is caused by *B. burgdorferi* sensu lato (s.l). It is currently the most important vector-borne disease in Europe[8,22,25]. Clinical signs of Lyme borreliosis include skin lesions and neurological symptoms[25,26].

*Babesia* spp. causes babesiosis which is a worldwide tick-borne hemoprotozoosis. *B. divergens* causes babesiosis in cattle but also in humans. *B. venatorum* sp1 (EU) is also capable of causing human babesiosis. However, discovering *B. venatorum* sp1 (EU) in Dutch ticks is a new development[21,22,27].

Granulocytic anaplasmosis and Tick borne fever are caused by *Anaplasma* spp., in particular *A. phagocytophilum* and *A. marginale.* Anaplasmosis can be seen in dogs, ruminants, horses and humans. I*. ricinus* is the main vector of *A. phagocytophilum*[21,22].

## Aim of the study

The main goal of this study is to research what role birds play in disseminating ticks and tick-borne pathogens. The pathogens carried by ticks can be of veterinary and medical importance[8,21,22,25]. The emphasis will be on the introduction of exotic ticks and exotic pathogens. It is therefore important to determine if there are other tick species than *I. ricinus* found on migratory birds and what pathogens these ticks carry.

## Materials and methods

### Bird capture and tick collection procedure

Ticks were collected from captured birds by Bert Winters, a field biologist involved in bird migration. Birds were captured with nets, which may only be used with a permit; they were removed from the nets by hand. The main reason for capturing birds was to ring them; at the same time the birds were measured, weighted and checked for ticks. Birds with ticks had the ticks removed with small tweezers. Collection locations were the water pump station Leemans: Noorderdijkweg 28, Wieringerwerf. And the Voorboezem: Molenweg 3, Hippolytushoef, both locations in North Holland. Ticks were collected from November 2013 till December 2014. Information sent with the ticks included host species, sex and age, number of ticks on the host and date and location of collection. Ticks were sent to the UCTD without preservation in 70% ethanol.

### Tick identification

Tick identification was done using a stereo microscope; genus, species and stage were specified. The book 'Ticks of domestic animals in the Mediterranean region: A guide to identification of species' provided pictures and information for clarification[12]. The emphasis was put on recognizing ticks of the genus *Ixodes*, in particular *I. ricinus* because this is the most abundant tick species in Europe[9,13,14,21–23].

### DNA extraction

A total of 191 ticks were subject to DNA extraction. Eighteen were adult ticks, 173 were nymphs. Larvae were not used in this survey. One sample is from one bird, one sample can be comprised of multiple ticks, taken from the same bird. A maximum of ten ticks per sample was taken to avoid blocking of spin columns during the DNA extraction. Adult ticks and exotic tick species such as *Hyalomma* spp. were tested separately.

For DNA extraction the Nucleo®Spin Tissue kit was used[28]. Ticks were put in 180 µl lysis buffer and after fifteen minutes at -80˚C a 5 mm metal bead was added. Disruption of the samples was done by the TissueLyser LT. The Utrecht Centre for Tick-borne Diseases (UCTD) protocol was used. See Appendix A for the protocol.

### Polymerase Chain Reaction (PCR)

| **PCR primers** | | |
|---|---|---|
| **Primer** | **Sequence** | **T*m* (˚C)** |
| Ehr-F | 5'- GGA ATT CAG AGT TGG ATC MTG GYT CAG - 3' | 61.0 |
| Ehr-R | 5' - Biotin - CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT - 3' | 69.5 |
| RLB-F2 | 5' - GAC ACA GGG AGG TAG TGA CAA G - 3' | 57.9 |
| RLB-R2 | 5'- Biotin - CTA AGA ATT TCA CCT CTG ACA GT - 3' | 53.7 |
| Bor-F | 5'- ACC ATA GAC TCT TAT TAC TTT GAC CA - 3' | 60.3 |
| Bor-R | 5'- Biotin – GAG AGT AGG TTA TTG GCC AGG G - 3' | 65.0 |

Table 2: Used PCR primers

Ehr-primers: *Anaplasma/Ehrlichia*
RLB2-primers: *Babesia/Theileria*
Bor-primers: *Borrelia*

The next step was a Polymerase Chain Reaction (PCR). The PCR was used to amplify specific DNA fragments. A master mix was made containing $H_2O$, 5x phire reaction buffer, dNTPs, forward primer, reverse primer and polymerase. After making the master mix a DNA sample was added after which the samples were put in the PCR thermocycler.

Primers of *Anaplasma/Ehrlichia, Babesia/Theileria* and *Borrelia* were used. Also a positive and negative PCR control was made to make sure that everything was working properly.

For the protocol used see Appendix B

| **PCR master mix. Reagents for 1 reaction** | |
|---|---|
| 15.875 µl | PCR grade $H_2O$ |
| 5.0 µl | 5x Phire reaction buffer |
| 0.5 µl | 10 mM dNTPs |
| 0.5 µl | Forward primer (20 pmol/µl) |
| 0.5 µl | Reverse primer (20 pmol/µl) |
| 0.125 µl | 2U/µl Phire Hot Start II DNA Polymerase |
| 2.5 µl | DNA sample |

Table 3: Reagents required for master mix

## Reverse line blot hybridization (RLB)

With the Reverse Line Blot Hybridization (RLB) it was possible to analyse multiple samples at the same time. The RLB assay used multiple species-specific oligonucleotide probes to detect and differentiate pathogens [29,30]. In this survey the samples were tested for *Anaplasma/Ehrlichia, Babesia/Theileria* and *Borrelia*. For the protocol used see Appendix C

# Results

## Tick collection and identification

A total of 191 ticks was used in this survey. Eighteen adults of which fourteen female and four male. The rest were nymphs. Identification showed that all but one were of the species *Ixodes ricinus,* the other was a female *Hyalomma scupense.*

The areas were the ticks were collected were the water pump station Leemans and the Voorboezem. The area of the water pump station has deciduous trees with water and a field with bushes. The Voorboezem is a deciduous forest. Nine of the 191 samples were taken at the Voorboezem.

Figure 1: Water pump station Leemans

## RLB results

| Bird species | Number of birds sampled | Tick stages | |
|---|---|---|---|
| | | Adult | Nymph |
| Blue throat (*Luscinia svecica*) | 1 | - | 1 |
| Common whitethroat (*Sylvia communis*) | 1 | ♀ 1 | - |
| Eurasian blackcap (*Sylvia atricapilla*) | 16 | - | 16 |
| Common blackbird *(Turdus merula*) | 44 | ♀ 9 | 35 |
| Great tit (*Parus major*) | 27 | ♀ 1; ♂ 1 | 25 |
| Eurasian bleu tit (*Cyanistes caeruleus*) | 1 | - | 1 |
| Treecreeper (*Certhia familiaris*) | 1 | - | 1 |
| European greenfinch (*Chloris chloris*) | 1 | - | 1 |
| Chaffinch (*Fringilla coelebs*) | 1 | - | 1 |
| Redwing (*Turdus iliacus*) | 8 | - | 8 |
| Song thrush (*Turdus philomelos*) | 26 | ♂ 1 | 25 |
| European robin (*Erithacus rubecula*) | 35 | ♂ 1 | 34 |
| Dunnock (*Prunella modularis*) | 16 | ♀ 1 | 15 |
| Eurasian wren (*Troglodytes troglodytes*) | 11 | ♀ 2; ♂ 1 | 8 |
| Hawfinch (*Coccothraustes coccothraustes*) | 2 | - | 2 |
| Total: 15 | 191 | 18 | 172 |

Table 4: Total number of birds from each species and stage. One sample can consist of multiple ticks.

**RLB batch 1**

Sample number 144 (2013) from a Blue tit (*Cyanistes caeruleus*), caught on 23-11-2013 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Theileria/Babesia* catch-all.

Sample number 38A from a Blackbird (*Turdus merula*), caught on 23-03-2014 at Leemans Gemaal was an *I. ricinus* female. It was positive for *Borrelia burgdorferi sensu lato* and *Borrelia garinii*.

Sample number 39 from a Dunnock (*Prunella modularis*), caught on 23-03-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Theileria/Babesia* catch-all and *Theileria equi*.

Sample number 40A from a Dunnock, caught on 23-03-2014 at Leemans Gemaal were 6 (pooled) *I. ricinus* nymphs. They were positive for *Theileria/Babesia* catch-all, *Babesia* catch-all 1, *Babesia divergens* and *Babesia venatorum* (sp EU 1).

Sample number 64 from a Common whitethroat (*Sylvia communis*), caught on 29-04-2014 at Leemans Gemaal was a *Hyalomma Scupense* female. It was positive for *Rickettsia* catch-all

Sample number 87 from a Dunnock, caught on 07-06-2014 at Leemans Gemaal were 10 out of 25 (pooled) *I. ricinus* nymphs. They were positive for *Rickettsia helvetica*.

Sample number 96 from a Blue throat (*Luscinia svecica*), caught on 14-06-2014 at Voorboezem was an *I. ricinus* nymph. It was positive for *Rickettsia helvetica*.

Sample number 99 from a Dunnock, caught on 16-06-2014 at Leemans Gemaal were 2 *I. ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 110 from a Dunnock, caught on 21-06-2014 at Leemans Gemaal were 10 *I. ricinus* nymphs. They were positive for *Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 113 from a Dunnock, caught on 21-06-2014 at Leemans Gemaal were 2 *I. ricinus* nymphs. They were positive for *Borrelia afzelii.*

Sample number 118 from a Dunnock, caught on 21-06-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Babesia* catch-all.

Sample number 121 from a Dunnock, caught on 21-06-2014 at Leemans Gemaal were 6 *I. ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all.

Sample number 133 from a Eurasian wren caught on 25-06-2014 at Leemans Gemaal was an *I. ricinus* female. It was positive for *Ehrlichia/Anaplasma* catch-all, *Theileria/Babesia* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 135 from a Dunnock, caught on 25-06-2014 at Leemans Gemaal were 2 *I. ricinus* nymphs. They were positive for *Borrelia burgdorferi* sensu lato and *Borrelia afzelii.*

Sample number 140 from a Great tit (*Parus major*), caught on 28-06-2014 at Leemans Gemaal was an *I. ricinus* male. It was positive for *Ehrlichia/Anaplasma* catch-all and *Ehrlichia ruminantium*.

Sample number 145 from a Eurasian wren, caught on 30-06-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Borrelia burgdorferi* sensu lato, *Borrelia garinii* and *Borrelia valaisiana.*

Sample number 146 from a Eurasian wren, caught on 07-07-2014 at Leemans Gemaal was an *I. ricinus* female. It was positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all, *Rickettsia helvetica*, and *Rickettsia massiliae*.

Sample number 146A from a Eurasian wren, caught on 07-07-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Theileria/Babesia* catch-all.

Sample number 152 from a Eurasian wren, caught on 07-07-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Borrelia burgdorferi* sensu lato and *Borrelia valaisiana.*

Sample number 182 from a European robin (*Erithacus rubecula*), caught on 17-07-2014 at Leemans Gemaal was an *I. ricinus* male. It was positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia massiliae*.

Sample number 194 from a Blackbird, caught on 10-08-2014 at Leemans Gemaal was an *I. ricinus* female. It was positive for *Ehrlichia/Anaplasma* catch-all, *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato.

Sample number 223 from a Blackbird, caught on 08-09-2014 at Leemans Gemaal was an *I. ricinus* female. It was positive for *Ehrlichia/Anaplasma* catch-all and *Theileria/Babesia* catch-all.

Sample number 225 from a Blackbird, caught on 13-09-2014 at Leemans Gemaal was an *I. ricinus* female. It was positive for *Ehrlichia/Anaplasma* catch-all.

Sample number 225(a) from a Blackbird, caught on 13-09-2014 at Leemans Gemaal was an *I. ricinus* female. It was positive for *Ehrlichia/Anaplasma* catch-all.

Sample number 250 from a Blackbird, caught on 11-10-2014 at Leemans Gemaal was an *I. ricinus* female. It was positive for *Ehrlichia/Anaplasma* catch-all.

Sample numbers 159, 6, 43, 62, 66A, 74, 100, 129, 133(a), 133A, 139, 147, 149, 248 and 250(a) were negative for every pathogen.

**RLB batch 2**

Sample number 42 from a Song thrush, caught on 05-04-2014 at Leemans Gemaal were 3 *Ixodes ricinus* nymphs. They were positive for *Borrelia afzelii.*

Sample number 55 from a Eurasian blackcap (*Sylvia atricapilla*), caught on 12-04-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica*.

Sample number 56 from a Song thrush (*Turdus philomelos*), caught on 22-04-2014 at Leemans Gemaal were 10 out of 17 *I. ricinus* nymphs. They were positive for *Babesia* catch-all 1, *Rickettsia helvetica* and *Borrelia valaisiana*.

Sample number 68 from a Song thrush, caught on 10-05-2014 at Leemans Gemaal were 8 *I. ricinus* nymphs. They were positive for *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva*

Sample number 79 from a Eurasian blackcap, caught on 02-06-2014 at Leemans Gemaal were 6 *I. ricinus* nymphs. They were positive for *Rickettsia helvetica*.

Sample number 82 from a Song thrush, caught on 02-06-2014 at Leemans Gemaal were 10 out of 19 *I. ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma catch-all, Theileria/Babesia*

*catch-all, Theileria catch-all and Theileria parva, Rickettsia helvetica, Borrelia garinii and Borrelia valaisiana.*

Sample number 84 from a Eurasian blackcap, caught on 07-06-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all, *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva*, *Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 88 from a Eurasian blackcap, caught on 07-06-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 89 from a Song thrush, caught on 07-06-2014 at Leemans Gemaal were 10 out of 18 *I. ricinus* nymphs. They were positive for *Borrelia burgdorferi sensu lato, Borrelia garinii and Borrelia valaisiana.*

Sample number 103 from a Eurasian blackcap, caught on 16-06-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva*.

Sample number 116 from a Eurasian blackcap, caught on 21-06-2014 at Leemans Gemaal were 2 *I. ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Anaplasma phagocytophilum*, *Theileria/Babesia* catch-all, *Babesia* catch-all 1, *Babesia divergens, Babesia venatorum* (sp EU 1) and *Rickettsia helvetica.*

Sample number 123 from a Song thrush, caught on 22-06-2014 at Voorboezem were 4 *I. ricinus* nymphs. They were positive for *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva*.

Sample number 124 from a Song thrush, caught on 22-06-2014 at Voorboezem was an *I. ricinus* nymph. It was positive for *Borrelia garinii.*

Sample number 125 from a Song thrush, caught on 22-06-2014 at Voorboezem was an *I. ricinus* nymph. It was positive for *Theileria/Babesia* catch-all, *Theileria* catch-all *Theileria parva* and *Borrelia garinii.*

Sample number 138 from a Eurasian blackcap, caught on 25-06-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all and *Anaplasma phagocytophilum.*

Sample number 160 from a Eurasian blackcap, caught on 07-07-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva*.

Sample number 171 from a Dunnock (*Prunella modularis*), caught on 14-07-2014 at Leemans Gemaal were 2 *I. ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Ehrlichia canis*, *Theileria/Babesia* catch-all, *Theileria* catch-all, *Theileria parva* and *Borrelia garinii.*

Sample number 185 from a Dunnock, caught on 21-07-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva*.

Sample number 199 from a Dunnock, caught on 10-08-2014 at Leemans Gemaal were 9 *I. ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Theileria* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 224 from a Eurasian wren, caught on 08-09-2014 at Leemans Gemaal were 2 *I. ricinus* nymphs. They were positive for *Babesia* catch-all 1.

Sample number 260 from a redwing, caught on 27-10-2014 at Leemans Gemaal were 2 *I. ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all and *Anaplasma phagocytophilum.*

Sample number 152 (2013), 160 (2013), 63, 66, 91, 97, 115, 117, 131, 137, 215, 239, 263, 279, 285, 4 (2014), 10 (2015), 11 (2015), 17 (2015) were negative for every pathogen.

**RLB Batch 3**

Sample number 142 from a Blackbird (*Turdus merula*), caught on 23-11-2013 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all

Sample number 148 from a Blackbird , caught on 24-11-2013 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Theileria/Babesia* catch-all, *Babesia* catch-all 1 and *Babesia venatorum (sp EU1)*.

Sample number 157 from a Blackbird , caught on 13-12-2013 at Leemans Gemaal were 2 *I. ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica.*

Sample number 44 from a European robin (*Erithacus rubecula*), caught on 05-04-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Theileria/Babesia* catch-all, *Babesia* catch-all 1 and *Babesia venatorum (sp EU1)*.

Sample number 47 from a European robin, caught on 06-04-2014 at Leemans Gemaal were 2 *I. ricinus* nymphs. They were positive for *Theileria/Babesia* catch-all and *Theileria equi*.

Sample number 191 from a Song thrush, caught on 10-08-2014 at Leemans Gemaal was were 2 *I. ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all

Sample number 238 from a Song thrush, caught on 05-10-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Theileria/Babesia* catch-all.

Sample number 241 from a Song thrush, caught on 10-10-2014 at Leemans Gemaal was an *I. ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica.*

Sample numbers 143 (2013), 145 (2013), 146 (2013), 147 (2013), 149 (2013), 150 (2013), 153 (2013), 155 (2013), 158 (2013), 21, 41, 67, 75, 78, 85, 90, 92, 93, 148, 161, 162, 168, 169, 174, 180, 188, 197, 200, 203, 232, 236, 242 were negative for every pathogen.

**RLB Batch 4**

Sample number 4 from a Blackbird, caught on 26-01-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Ehrlichia/Anaplasma catch-all* and *Ehrlichia ruminantium.*

Sample number 10 from a Blackbird, caught on 26-02-2014 at Leemans Gemaal were 10 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Theileria/Babesia* catch-all, *Babesia* catch-all 1 and *Babesia venatorum (sp EU 1).*

Sample number 11 from a Blackbird, caught on 01-03-2014 at Leemans Gemaal were 7 *Ixodes ricinus* nymphs. They were positive for *Theileria/Babesia* catch-all, *Babesia* catch-all 1 and *Babesia venatorum* (sp EU 1).

Sample number 15 from a Great tit, caught on 01-03-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Ehrlichia/Anaplasma catch-all* and *Rickettsia helvetica.*

Sample number 106 from a European robin, caught on 16-06-2014 at Leemans Gemaal were 2 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all*, Theileria/Babesia* catch-all, and *Rickettsia helvetica.*

Sample number 108 from a European robin, caught on 21-06-2014 at Leemans Gemaal were 4 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all.

Sample number 111 from a European robin, caught on 21-06-2014 at Leemans Gemaal were 6 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica.*

Sample number 134 from a European robin, caught on 25-06-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Babesia* catch-all 1.

Sample number 142 from a European robin, caught on 28-06-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Rickettsia helvetica.*

Sample number 155 from a European robin, caught on 07-07-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all*, Rickettsia catch-all* and *Rickettsia helvetica.*

Sample number 176 from a European robin, caught on 15-07-2014 at Leemans Gemaal were 2 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica.*

Sample number 181 from a European robin, caught on 17-07-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all.

Sample number 182A from a European robin, caught on 17-07-2014 at Leemans Gemaal were 3 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all.

Sample number 222 from a European robin, caught on 08-09-2014 at Leemans Gemaal were 3 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all.

Sample number 227 from a European robin, caught on 13-09-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all and *Anaplasma phagocytophilum.*

Sample number 247 from a European robin, caught on 10-10-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all and *Anaplasma phagocytophilum, Rickettsia* catch-all and *Rickettsia helvetica.*

Sample numbers 1, 2, 3, 5, 7, 8, 9, 12, 13, 16, 17, 18, 19, 101, 102, 112, 128, 132, 151, 156, 157, 208, 209 and 212 were negative for every pathogen.

**RLB Batch 5**

Sample number 20 from a Great tit, caught on 06-03-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica.*

Sample number 22 from a Great tit, caught on 06-03-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all, *Neoehrlichia mikurensis, Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 24 from a Blackbird, caught on 06-03-2014 at Leemans Gemaal were 5 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 35 from a Blackbird, caught on 17-03-2014 at Leemans Gemaal were 10 out of 36 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Neoehrlichia mikurensis*, *Borrelia burgdorferi* sensu lato and *Borrelia garinii.*

Sample number 37 from a Great tit, caught on 22-03-2014 at Leemans Gemaal were 3 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 38 from a Blackbird, caught on 23-03-2014 at Leemans Gemaal were 2 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Theileria* catch-all, *Borrelia garinii, Borrelia valaisiana, Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 46 from a Blackbird, caught on 06-04-2014 at Leemans Gemaal was an. It was *Ixodes ricinus* nymph. It was positive for *Theileria* catch-all and *Rickettsia helvetica.*

Sample number 52 from a Blackbird, caught on 12-04-2014 at Leemans Gemaal were 2 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Neoehrlichia mikurensis, Borrelia valaisiana, Rickettsia* catch-all and *Rickettsia helvetica.*

Sample number 61 from a Blackbird, caught on 25-04-2014 at Leemans Gemaal were 10 out of 24 *Ixodes ricinus* nymphs. They were positive for *Borrelia burgdorferi* sensu lato and *Borrelia valaisiana.*

Sample number 77 from a Blackbird, caught on 01-06-2014 at Voorboezem were 10 *Ixodes ricinus* nymphs. They were positive for *Borrelia valaisiana.*

Sample number 80A from a Blackbird, caught on 02-06-2014 at Leemans Gemaal were 10 out of 11 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Borrelia burgdorferi* sensu lato, *Borrelia garinii, Borrelia valaisiana* and *Rickettsia helvetica.*

Sample number 95 from a Blackbird, caught on 14-06-2014 at Voorboezem were 8 *Ixodes ricinus* nymphs. They were positive for *Borrelia burgdorferi* sensu lato and *Borrelia valaisiana.*

Sample number 105 from a Blackbird, caught on 16-06-2014 at Leemans Gemaal were 10 out of 14 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Borrelia burgdorferi* sensu lato, *Borrelia garinii* and *Rickettsia helvetica.*

Sample number 114 from a Blackbird, caught on 21-06-2014 at Leemans Gemaal were 10 out of 13 *Ixodes ricinus* nymphs. They were positive for *Theileria* catch-all, *Borrelia burgdorferi* sensu lato, *Borrelia garinii* and *Borrelia valaisiana.*

Sample number 122 from a Blackbird, caught on 21-06-2014 at Leemans Gemaal were 10 out of 11 *Ixodes ricinus* nymphs. They were positive for *Ehrlichia/Anaplasma* catch-all, *Borrelia burgdorferi* sensu lato, *Borrelia garinii, Borrelia valaisiana* and *Rickettsia helvetica*.

Sample number 218 from a Great tit, caught on 28-08-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph. It was positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch/all and *Rickettsia helvetica*.

Sample number 251 from a Great tit, caught on 13-10-2014 at Leemans Gemaal was an *Ixodes ricinus* nymph . It was positive for *Theileria/Babesia* catch-all and *Theileria equi*.

Sample numbers 48, 49, 50, 51, 53, 57, 65, 71, 140A, 175, 230, 163, 234 and 253 were negative for every pathogen.

| | ***Total number of ticks*** | ***Percentage (%)*** | ***Adults*** | ***%*** | ***Nymphs*** | ***%*** |
|---|---|---|---|---|---|---|
| | 191 | 100% | 18 | 100 % | 173 | 100% |
| ***Pathogen*** | | | | | | |
| *Ehrlichia/Anaplasma* catch-all | 48 | 25,1 % | 9 | 50 % | 39 | 22,5 % |
| *Anaplasma phagocytophilum* | 6 | 3,1 % | 1 | 5,6 % | 5 | 2,9% |
| *Ehrlichia ruminantium* | 2 | 1,0 % | 1 | 5,6 % | 1 | 0,6 % |
| *Ehrlichia canis* | 1 | 0,5 % | 0 | 0 % | 1 | 0,6 % |
| *Neoehrlichia mikurensis* | 3 | 1,6 % | - | - | 3 | 1,7% |
| *Theileria/Babesia* catch-all | 25 | 13,1 % | 4 | 22,2% | 21 | 12,1 % |
| *Babesia* catch-all 1 | 8 | 4,2 % | 0 | 0 % | 8 | 4,6 % |
| *Babesia divergens* | 2 | 1,0 % | 0 | 0 % | 2 | 1,2 % |
| *Babesia venatorum* (sp EU1) | 6 | 3,1 % | 0 | 0 % | 6 | 3,5 % |
| *Theileria* catch-all | 13 | 6,8 % | - | - | 13 | 7,5 % |
| *Theileria equi* | 3 | 1,6 % | 1 | 5,6 % | 2 | 1,2 % |
| *Theileria parva* | 9 | 4,7 % | - | - | 9 | 5,2 % |
| *Borrelia burgdorferi s.l* | 18 | 9,4 % | 2 | 11,1% | 16 | 9,2 % |
| *Borrelia garinii* | 15 | 7,9 % | 1 | 5,6 % | 14 | 8,1 % |
| *Borrelia afzelii* | 5 | 2,6 % | 0 | 0 % | 5 | 2,9 % |
| *Borrelia valaisiana* | 15 | 7,9 % | 0 | 0 % | 15 | 8,7 % |
| *Rickettsia* catch-all | 17 | 8,9 % | 4 | 22,2% | 13 | 7,5 % |
| *Rickettsia helvetica* | 33 | 17,3 % | 2 | 11,1% | 31 | 17,9 % |
| *Rickettsia massiliae* | 2 | 1,0 % | 2 | 11,1% | 0 | 0 % |

Table 5: Pathogens found in absolute numbers and prevalence

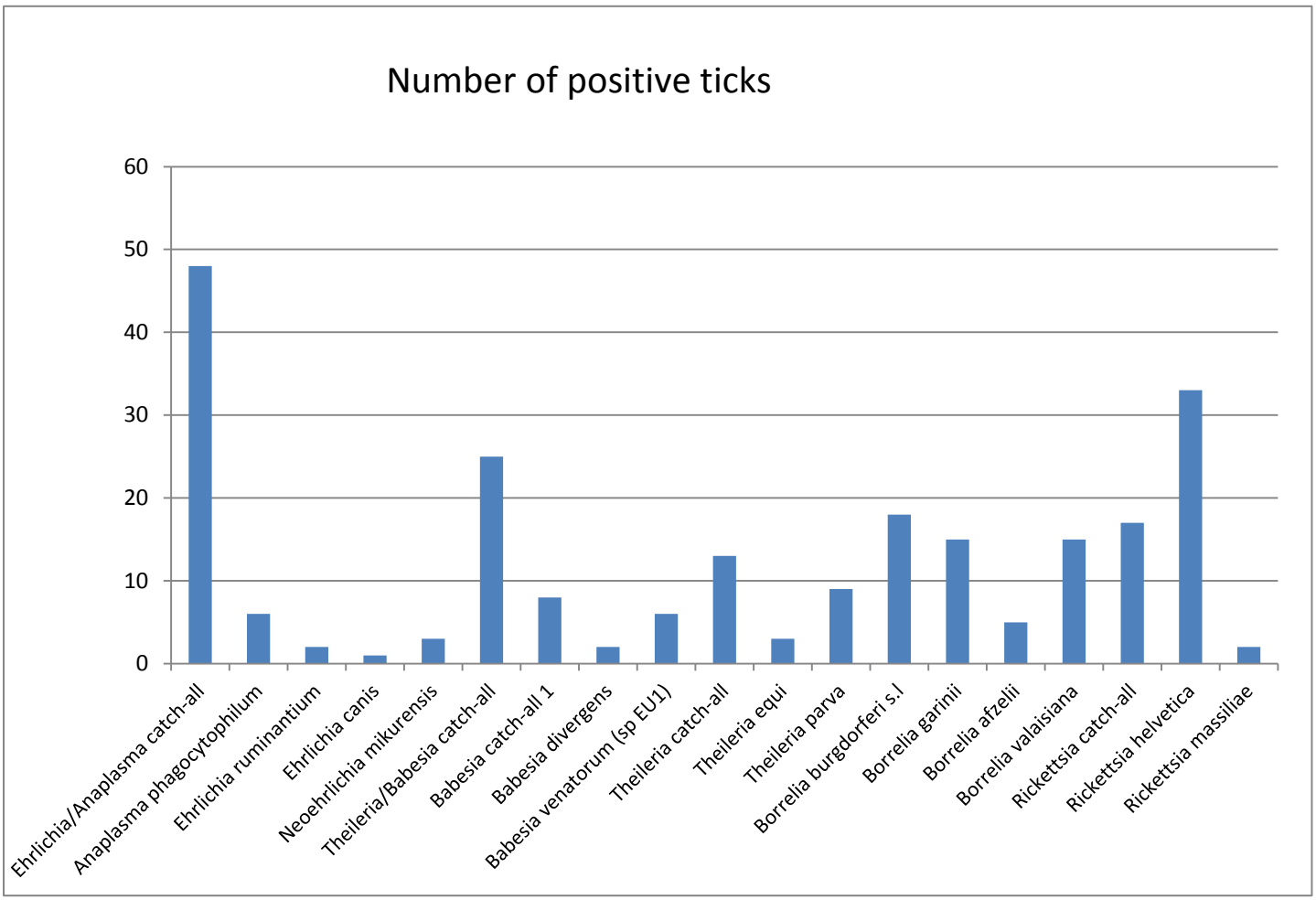


Figure 2: Number of positive ticks for a particular pathogen

## Discussion

Migration is a well-researched phenomenon. With the use of ringing data a lot of information is available[4–6]. Available data from ringing is important in the research of migration. Research is difficult because many bird species are so small but new technological devices are under development. However, the impact of such devices is not yet studied. For now ringing data has showed its usefulness in migration research[4].

As was the expectation, most ticks found were of the species *Ixodes ricinus*. This was expected, because *I. ricinus* is the most abundant tick in Europe[9,13,14,21–24].

One tick found was a female *Hyalomma scupense*, this tick is widespread in southern Europe. However, it is known as a tick mostly found on cattle, not on birds[31,32]. This was not expected and may mean that birds do have a role in disseminating certain tick species that are now only known as having other hosts. Only one other tick (*Hyalomma*) was found in this survey, so further research is necessary.

A total of five batches of DNA extractions were done. PCR and RLB were done in batches of 40 samples, except for the last one. A positive and negative PCR control was added.

The RLB blot had a maximum of 45 slots. With a positive PCR control and two RLB controls this meant that 40 samples per blot were possible, for the first and last slot were filled with buffer. The negative PCR control was not used.

During the survey problems arose in the lab. A contamination with *Theileria parva* was observed, measures were taken to make sure the contamination was under control. The DNA extraction lab and RLB lab surfaces were cleaned with sodiumhypochloride and the ground was cleaned with chlorine. All opened and used supplies and buffers were replaced with new ones/unused/unopened. After that *Theileria parva* was not observed anymore.

At a certain point the positive RLB controls used were almost empty, therefore they were diluted. After that the signals on the RLB were very weak. Mixing the samples before pipetting in the Eppendorf cups made sure that the chance of pipetting DNA was as high as possible.

In RLB batches 2,3,4 and 5 the *Borrelia* positive PCR controls were not present. For RLB batch 5 the *Anaplasma/Theileria* positive PCR control was also not present. This meant that the *Borrelia* PCR of batch 2 till 4 had to be done again. The PCR and RLB of batch 5 had to be done all over again.

Despite the lack of positive *Borrelia* PCR controls in batch 3 and 4 during the first RLB there were *Borrelia* signals present. The same happened again in the second RLB of batch 5, no *Borrelia* PCR controls but there were *Borrelia* signals in the samples. The second RLB of batch 3 had no signals at all, this one is not included in the appendix.

To test that the PCR controls were not the problem a PCR agarose gel of all the controls was made. This showed that they were not the problem.

Two more DNA extractions were done, along with two more PCR's and RLB's. These results are not included in this report because the results were inconclusive. One RLB showed no results at all, except for the positive RLB controls. The other showed too much signals, almost all samples had catch-all signals of *Anaplasma, Theileria* and *Babesia*.

This problem was noticed, not only in these results, but also in other results.

It was agreed to clean the labs again. Again all the supplies and buffers were replaced by new ones. Not only the DNA extraction lab and the RLB lab were cleaned this time, also the PCR lab was cleaned with sodiumhypochloride and the floor was mobbed with chlorine. The floor between the labs was also mobbed.

Lab coats were cleaned at 90 degrees.

After this the results were still abnormal. It was decided that new PCR controls had to be introduced. Instead of *Anaplasma phagocytophilum, Theileria equi* , *Borrelia afzellii* and *B. valaisiana, Theileria parva, Ehrlichia ruminantium* and *Borrelia burgdorferi* (Ameland) were taken for the positive PCR controls. These had to be tested to make sure they were suitable.

A DNA extraction, PCR and RLB of the new controls was done. The results of this RLB were still inconclusive. Not only *Theileria parva, Ehrlichia ruminantium* and *Borrelia burgdorferi* (Ameland) but

also *Anaplasma marginale, A. central, Babesia bigemina, B. canis, B canis canis, B major, B vogeli, Ehrlichia canis, E. chaffeensis* and *Theileria annulata* were tested in this RLB. These pathogens were in stock and to make sure there were working PCR controls they were all tested. There was not enough *Borrelia burgdorferi* in stock to use so therefore it was decided to use the former *Borrelia* control, there was still enough present in the freezer. It only had to be diluted.

Membranes 1,2,3 and 4 were not to be used anymore for the RLB, only membranes 5 and 6 could be used. Membranes 5 and 6 were relatively new and to make sure the membranes were not the problem only those could be used.

The protocol for the RLB was adjusted. Rather than cleaning the workspace with ethanol it had to be cleaned with sodiumhypochloride. This to prevent possible fixation of DNA by ethanol.

An external professional came by to make recommendations about the lab and how to prevent future inconclusive results. The polymerase used in the lab was tested against a new, more specific polymerase. The results showed that the new polymerase gave no signals on the RLB, while the old polymerase gave, again, too many signals.

Lab coats were cleaned again, but now they were washed in chlorine and after that washed at 90 degrees. Also the idea of using colour codes for the different labs was introduced. The external professional also advised to take automatic door closers. These measures were suggested to reduce the chance of contamination (of other labs).

A protocol for lab routes was suggested, also to reduce the chance of contamination.

## Results

The results show multiple catch-all signals of multiple pathogens without any result for a specific species of that pathogen. For instance, a catch-all for *Ehrlichia/Anaplasma* without a signal for *A. phagocytophilum* or any other. The species of that pathogen was either not on the membrane used or it can indicate that there is a new (undiscovered) species of that pathogen present in the sample.

The prevalence of *Ehrlichia/Anaplasma* species in adult ticks was 50% in this survey, in another study a prevalence of 35% was observed[26]. Because of the low number of adult ticks, 18, the prevalence observed in this survey is not a good representation of reality.

*Ehrlichia canis* was detected in one sample, however, *I. ricinus* does not transmit *E. canis*. The tick that does is the brown dog tick *Ripicephalus sanguineus*[24,33] Because *I. ricinus* cannot transmit this pathogen the finding is not of interest to us in this survey.

*Rickettsia massiliae* is recognized as a human pathogen and can cause spotted fever. *Rickettsia helvetica* causes mild illness with among other things headache and myalgia, these symptoms disappear spontaneously [34].

*Babesia divergens* was found in two samples, *Babesia venatorum* (sp EU1) was found in six samples, these pathogens can be transmitted to humans and can cause babesiosis. *I. ricinus* is considered as the main vector for transmission of *B. divergens* to humans. Transoverial transmission of *Babesia divergens* is described, this means that infected adult ticks may lay infected eggs [7,35]. In Europe this disease is relatively rare but can be lethal. The implications of this finding can be very serious [27]. If a tick, picked up by a bird in an area with *Babesia* spp., feeds and falls off in an area were *Babesia* spp. is not present and the tick reproduces and lays infected eggs that molt into nymphs, humans may get infected with, for example, *Babesia divergens.*

*Theileria equi* has been found in three samples. It can cause equine piroplasmosis, a disease that affects the immune system [27,33]. Equine piroplasmosis can be lethal for horses, treatment is costly and horses stay seropositive for life. It mainly can be found in tropical and subtropical regions [36]. *I. ricinus* is not considered to be a vector for equine piroplasmosis [37]. These results are not relevant for this survey, because transmission cannot occur.

*Neoerhlichia mikurensis* is a relatively new emerging tick-borne disease. In humans the symptoms are non-specific, which can account for the lack of patients. Most patients admitted were immune-compromised[38,39]. It has also been described in rodents and dogs. *I. ricinus* has been regarded as its main vector in the Netherlands. Studies showed varying prevalences between de 1

and 16% and even between 1 and 23 %[39,40]. The prevalence found in this survey was 1,7%, this is within the range found in other studies. However, more research is necessary.

## Conclusion

The most found tick species was *I. ricinus*. However, there was also a female *Hyalomma scupense* found. This means that birds can carry other tick species, but more research is necessary.

A broad array of pathogens was found in this survey, pathogens found include zoonotic pathogens. Although there were no exotic pathogens found, some are relatively new or can cause diseases that are not very common in Europe. The list of pathogens tested in this survey was not complete, in following surveys this list may be expanded to test for more and exotic pathogens.

Birds do have a role in the dissemination of ticks and tick-borne pathogens. Nevertheless more data is needed for subsequent research.

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## Appendix A: DNA extraction protocol

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

### DNA EXTRACTION FROM TICKS

| | |
|---|---|
| **Sample description** | |
| **Number of samples** | |

**Wear gloves and use filter pipet tips**

**Strictly follow the one-way route: Clean room → Dirty room → PCR room**

| | | Done |
|---|---|---|
| 1 | Clean workspace with sodium hypochlorite. | |
| 2 | Turn on a water bath at 56°C. | |
| 3 | Take the proteinase K solution from the freezer and store at 4°C. | |
| 4 | Wash the ticks in a sonofication bath with demineralized water for up to 30 seconds. | |
| 5 | Put the ticks, with cleaned forceps, in 1.5ml tubes with 70% ethanol and vortex for several seconds. | |
| 6 | Wash the forceps in 70% ethanol followed by washing in demineralized water after each tick. | |
| 7 | Take the ticks from the tubes and let it dry on a clean tissue paper and place the dried ticks in a sterile 2ml tube with 180µl T1 lysis buffer. | |
| 8 | Freeze the samples at -80°C for 15 minutes. | |
| 9 | Add a 5 or 7mm (depending on tick size) metal bead to the frozen samples. | |
| 10 | Disrupt the ticks in the TissueLyser LT at 50 oscillations per second for 3 minutes. | |
| 11 | Briefly spin down the tubes. **1000x g maximum!** | |
| 12 | Add 25µl proteinase K and vortex. | |
| 13 | Prelyse the samples at 56°C in a water bath for 3 hours and vortex every hour. | |
| 14 | **During the incubation**; empty and clean the sonification bath. | |
| 15 | **During the last incubation hour** ; turn on the heating block at 70°C and preheat the BE buffer. | |
| 16 | Briefly spin down the tubes. **1000x g maximum!** | |
| 17 | Add 200µl B3 buffer and vortex. | |
| 18 | Incubate the tubes at 70°C for 15 minutes. | |

| | | |
|---|---|---|
| 19 | Briefly spin down the tubes. **1000x g maximum!** | |
| 20 | Add 210µl 96% ethanol, vortex and briefly spin down the tubes. **1000x g maximum!** | |
| 21 | Transfer the supernatant to new sterile 1.5ml tubes. (Tick parts are allowed to be transferred.) | |
| 22 | Centrifuge the tubes at 11,000x g for 2 minutes. | |
| 23 | Transfer the supernatant to spin columns. Avoid pipetting tick parts, as it can block the spin column. | |
| 24 | Centrifuge the columns at 11,000x g for 1 minute. Discard the flow through. | |
| 25 | Add 500µl BW buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through. | |
| 26 | Add 600µl B5 buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through. | |
| 27 | Centrifuge the columns at 11,000x g for 1 minute. | |
| 28 | Place the spin columns in sterile 1.5ml tubes. **Label the tubes accordingly.** | |
| 29 | Add 100µl preheated BE buffer directly on the membrane of the spin columns and incubate at room temperature for 1 minute. | |
| 30 | Centrifuge the columns at 11,000x g for 1 minute. Discard the spin columns. | |
| 31 | Store the DNA samples at 4°C for use within the next few days or store at -20°C for long term preservation. | |
| 32 | Turn off all equipment and clean working space with sodium hypochlorite. | |

DNA extraction done:

by _____ on _____ _____

Signature

**Comments:**

## Appendix B: PCR amplification protocol

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

**PCR RLB PROCEDURE**

| | |
|---|---|
| **Sample description** | |
| **Number of samples** | |

**Wear (green) gloves and use filter pipet tips**

**Strictly follow the one-way route: Clean room → Dirty room → PCR room**

| **Primers:** | ***Anaplasma* *Ehrlichia*** | ***Babesia* *Theileria*** | ***Borrelia*** | ***Rickettsia*** | **Other:** |
|---|---|---|---|---|---|

| **Reagent** | **1x** | **Number of samples + 10%** | |
|---|---|---|---|
| PCR grade $H_2O$ | 15.875µl | | |
| 5x Phire reaction buffer | 5.0µl | | |
| 10mM dNTPs | 0.5µl | | |
| Forward primer (20pmol/µl) | 0.5µl | | |
| Reverse primer (20pmol/µl) | 0.5µl | | |
| 2U/µl Phire Hot Start II DNA polymerase | 0.125µl | | |

| | | **Done** |
|---|---|---|
| 1 | Put DNA samples a (few) day(s) before the PCR at 4°C. | |
| 2 | Turn on the DNA workstations in the clean room and the dirty room. | |
| 3 | Clean workspace in both DNA workstations with sodium hypochloride. | |
| 4 | Label the PCR and Eppendorf tubes and put them in the DNA workstation in the clean room | |
| 5 | Turn on the UV-light in both DNA workstations for 20 minutes. | |
| 6 | **During the UV-light**; thaw the PCR reagents at room temperature, except the polymerase. | |
| 7 | Prepare the PCR mix in the Eppendorf tube(s). Multiply the reagent volumes by the number of samples plus 10% of the number of samples: 40 DNA samples + 1 PCR control = 41 + 10% = 45 samples. | |

| | | | |
|---|---|---|---|
| 8 | Pipet the master mix gently up and down to mix well. | | |
| 9 | Pipet 22,5µl master mix to each PCR tube and add the leftover mix to an additional tube which will be the negative PCR control. | | |
| 10 | Close the PCR tubes and remove them from the workstation, clean the workspace with sodium hypochloride and turn on the UV-light for 20 minutes. | | |
| 11 | Take the closed PCR tubes to the dirty room and place them in the workstation. | | |
| 12 | Vortex the DNA samples, spin them down briefly at 11,000x g and place them in the workstation. | | |
| 13 | Add 2.5µl DNA sample to the corresponding PCR tube. | | |
| 14 | Add 2.5µl of the positive control (, corresponding to the PCR to be performed,) to the positive PCR control tube. | | |
| 15 | Vortex and spin down briefly. | | |
| 16 | Clean the workstation with sodium hypochloide and turn on the UV-light for 20 minutes. | | |
| 17 | Run the corresponding PCR program. | | |
| 18 | Store the PCR products at 4°C for use within the next few days or store at -20°C for long term preservation. | | |
| 19 | Turn off both DNA workstations after the UV-light is switched off. | | |

PCR done:

by _____ on _____ _____

Signature

**Comments:**

## Appendix C: RLB protocol

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

### REVERSE LINE BLOT HYBRIDIZATION PROCEDURE

| | |
|---|---|
| **Sample description** | |
| **Number of samples** | |
| **Membrane ID** | |

**Wear gloves and use non-filter pipet tips**

**Strictly follow the one-way route: Clean room → Dirty room → PCR room**

| | | Done |
|---|---|---|
| 1 | Clean workspace with 70% ethanol. | |
| 2 | Turn on a heating block at 100°C. | |
| 3 | Turn on the hybridization oven at 42°C en preheat 50ml 2x SSPE/0.5% SDS solution. | |
| 4 | Turn on the water bath at 50°C en preheat the bottle with 2x SSPE/0.5% SDS solution. | |
| 5 | Combine and dilute the PCR products per DNA sample in a 1.5ml tube. Take 10µl of every PCR product and add 2x SSPE/0.1% SDS to a final volume of 160µl. (10µl *Anaplasma/Ehrlichia* PCR + 10µl *Babesia/Theileria* PCR + 140µl 2x SSPE/0.1% SDS.) | |
| 6 | Take 10µl of the RLB positive controls and add 150µl 2x SSPE/0.1% SDS to a 1.5ml tube, | |
| 7 | Denature the diluted PCR samples and controls at 100°C for 10 minutes. | |
| 8 | **During the denaturation step**; wash the membrane at room temperature with 2X 2SSPE/0.1% SDS for 5 minutes under gentle shaking and fill a bucket with ice. | |
| 9 | Immediately transfer the samples in order on ice after the denaturation. | |
| 10 | Prepare the miniblotter by placing the membrane on the lanes, with the line pattern of the membrane perpendicular to the lanes of the blotter. Place de support cushion on the membrane followed by the other half of the blotter. Turn the blotter right-side up without moving the membrane and turn the screws hand-tight, | |
| 11 | Remove residual fluid in the slots by aspiration. | |
| 12 | Briefly spin down the tubes at 4°C and place them back on ice in order. | |
| 13 | Fill the slots with the samples (150µl) and fill the first, last and other empty slots with 2x SSPE/0.1% SDS. **Avoid air bubbles.** | |
| 14 | Hybridize the blotter at 42°C for 60 minutes in the hybridization oven without shaking. | |
| 15 | Remove the samples by aspiration. | |
| 16 | Dissemble the blotter and remove the membrane from the blotter. | |

| | | | |
|---|---|---|---|
| 17 | Wash the membrane twice with preheated 2x SSPE/0.5% SDS at 50°C for 10 minutes under gentle shaking. | | |
| 18 | **During the washing step**; clean the blotter and the support cushion. | | |
| 19 | Incubate the membrane with 50ml 2x SSPE/0.5% SDS + 5µl streptavidin at 42°C for 30 minutes in the hybridization oven under gentle shaking. **Discard the streptavidin solution in a tube and into the bio-waste bin. Do not pour it in the sink.** | | |
| 20 | **During the streptavidin hybridization**; change the water bath temperature to 42°C and preheat the bottle with 2x SSPE/0.5% SDS solution. Keep the lid open. | | |
| 21 | Wash the membrane twice with preheated 2x SSPE/0.5% SDS solution at 42°C for 10 minutes under gentle shaking. | | |
| 22 | Change the water bath temperature to 80°C and preheat the bottle with 1% SDS solution. | | |
| 23 | Wash the membrane twice with 2x SSPE at room temperature for 5 minutes, under gentle shaking. | | |
| 24 | **During the washing step**; prepare the foil and film cassette and check if the developing machine is on (5th floor). | | |
| 25 | Add 10ml ECL (5ml ECL1 + 5ml ECL2) to the membrane and gently shake by hand until the whole membrane is covered. **Discard the ECL in a tube and into the bio-waste bin. Do not pour it in the sink.** | | |
| 26 | Cover the membrane in foil and place it in the film cassette. **Avoid air bubbles.** | | |
| 27 | Go to the dark room and expose a film to the membrane for 10 minutes. | | |
| 28 | Develop the film with the developing machine. | | |
| 29 | Remove the foil and wash the membrane twice with preheated 1% SDS at 80°C for 30 minutes under gentle shaking. | | |
| 30 | Wash the membrane with 20mM EDTA at room temperature for 15 minutes under gentle shaking. | | |
| 31 | Store the membrane in a seal bag with 20mM EDTA at 4°C. | | |
| 32 | Turn off all equipment and clean workspace. | | |

RLB hybridization done:

by _____ on _____ _____

Signature

**Comments:**

## Appendix D: Sample information

**RLB 1**

| Ticknr | Detectiondate | number | Tick Species | Stage | Host | Location | RLB Results |
|---|---|---|---|---|---|---|---|
| 144 | 23-11-2013 | 1 | Ixodes Ricinus | Nymph | Blue tit | Leemans Gemaal | positive *Theileria/Babesia* catch-all |
| 159 | 20-12-2013 | 1 | I. Ricinus | Female | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 6 | 23-2-2014 | 1 | I. Ricinus | Female | Great tit | Leemans Gemaal | Negative for every pathogen |
| 38a | 23-3-2014 | 1 | I. Ricinus | Female | Common blackbird | Leemans Gemaal | positive *Borrelia burgdorferi sensu lato and Borrelia garinii* |
| 39 | 23-3-2014 | 1 | I. Ricinus | Female | Dunnock | Leemans Gemaal | positive *Theileria/Babesia* catch-all and *Theileria equi* |
| 40a | 23-3-2014 | 6 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Theileria/Babesia* catch-all, *Babesia* catch-all 1, *Babesia divergens* and *Babesia venatorum* (spEU1) |
| 43 | 5-4-2014 | 1 | I. Ricinus | Nymph | Redwing | Leemans Gemaal | Negative for every pathogen |
| 62 | 25-4-2014 | 1 | I. Ricinus | Nymph | Eurasian wren | Leemans Gemaal | positive *Theileria/Babesia* catch-all |
| 64 | 29-4-2014 | 1 | Hyalomma Scupense | Female | Common whitethroat | Leemans Gemaal | positive *Rickettsia* catch-all |
| 66a | 1-5-2014 | 1 | I. Ricinus | Male | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 74 | 23-5-2014 | 1 | I. Ricinus | Nymph | Eurasian wren | Leemans Gemaal | Negative for every pathogen |
| 87 | 7-6-2014 | 10 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Rickettsia helvetica* |
| 96 | 14-6-2014 | 1 | I. Ricinus | Nymph | Bluethroat | Voorboezem | positive *Rickettsia helvetica* |
| 99 | 16-6-2014 | 2 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica* |
| 100 | 16-6-2014 | 7 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | Negative for every pathogen |
| 110 | 21-6-2014 | 10 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Rickettsia* catch-all and *Rickettsia helvetica* |
| 113 | 21-6-2014 | 2 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Borrelia afzelii* |
| 118 | 21-6-2014 | 1 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Theileria/Babesia* catch-all |
| 121 | 21-6-2014 | 6 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all |
| 129 | 24-6-2014 | 2 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | Negative for every pathogen |
| 133 | 25-6-2014 | 1 | I. Ricinus | Female | Eurasian wren | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Theileria/Babesia* catch-all, *Rickettsia* catch-all and *Rickettsiahelvetica* |
| 133(a) | 25-6-2014 | 1 | I. Ricinus | Male | Eurasian wren | Leemans Gemaal | Negative for every pathogen |
| 133a | 25-6-2014 | 2 | I. Ricinus | Nymph | Eurasian wren | Leemans Gemaal | Negative for every pathogen |
| 135 | 25-6-2014 | 4 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Borrelia burgdorferi sensu lato* and *Borrelia afzelii* |
| 139 | 25-6-2014 | 3 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | Negative for every pathogen |
| 140 | 28-6-2014 | 2 | I. Ricinus | Male | Great tit | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all and *Ehrlichia ruminantium* |
| 145 | 30-6-2014 | 2 | I. Ricinus | Nymph | Eurasian wren | Leemans Gemaal | positive *Borrelia burgdorferi* sensu lato, *Borrelia garinii* and *Borrelia valaisiana* |
| 146 | 7-7-2014 | 2 | I. Ricinus | Female | Eurasian wren | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all, *Rickettsia helvetica* and *Rickettsia massiliae* |
| 146a | 7-7-2014 | 1 | I. Ricinus | Nymph | Eurasian wren | Leemans Gemaal | positive *Theileria/Babesia* catch-all |
| 147 | 7-7-2014 | 1 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | Negative for every pathogen |
| 149 | 7-7-2014 | 1 | I. Ricinus | Nymph | European greenfinch | Leemans Gemaal | Negative for every pathogen |
| 152 | 7-7-2014 | 2 | I. Ricinus | Nymph | Eurasian wren | Leemans Gemaal | positive *Borrelia burgdorferi* sens lato and *Borrelia valaisiana* |
| 182 | 17-7-2014 | 1 | I. Ricinus | Male | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia massiliae* |
| 194 | 10-8-2014 | 1 | I. Ricinus | Female | Common blackbird | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato |
| 223 | 8-9-2014 | 1 | I. Ricinus | Female | Common blackbird | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all and *Theileria/Babesia* catch-all |
| 225 | 13-9-2014 | 1 | I. Ricinus | Female | Common blackbird | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all |
| 225(a) | 13-9-2014 | 1 | I. Ricinus | Female | Common blackbird | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all |
| 248 | 11-10-2014 | 1 | I. Ricinus | Female | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 250 | 11-10-2014 | 1 | I. Ricinus | Female | Common blackbird | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all |
| 250(a) | 11-10-2014 | 1 | I. Ricinus | Female | Common blackbird | Leemans Gemaal | Negative for every pathogen |

**RLB 2**

| Ticknr | Detectiondate | number | Tick Species | Stage | Host | Location | RLB Results |
|---|---|---|---|---|---|---|---|
| 152 | 8-12-2013 | 2 | Ixodes Ricinus | Nymph | Hawfinch | Leemans Gemaal | Negative for every pathogen |
| 160 | 31-12-2013 | 1 | I. Ricinus | Nymph | Hawfinch | Leemans Gemaal | Negative for every pathogen |
| 42 | 5-4-2014 | 3 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | positive *Borrelia afzelii* |
| 55 | 12-4-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica*. |
| 56 | 22-4-2014 | 10 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | positive *Babesia* catch-all 1, *Rickettsia helvetica* and *Borrelia valaisiana* |
| 63 | 28-4-2014 | 9 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 66 | 1-5-2014 | 4 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 68 | 10-5-2014 | 8 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | positive *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva* |
| 79 | 2-6-2014 | 6 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | positive *Rickettsia helvetica*. |
| 82 | 2-6-2014 | 10 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva*, *Rickettsia helvetica, Borrelia garinii* and *Borrelia valaisiana.* |
| 84 | 7-6-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva* (contamination), *Rickettsia* catch-all and *Rickettsia helvetica.* |
| 88 | 7-6-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all and *Rickettsia* catch-all and *Rickettsia helvetica.* |
| 89 | 7-6-2014 | 10 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | positive *Borrelia burgdorferi* sensu lato, *Borrelia garinii* and *Borrelia valaisiana.* |
| 91 | 7-6-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | Negative for every pathogen |
| 97 | 16-6-2014 | 4 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 103 | 16-6-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | positive *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva* |
| 115 | 21-6-2014 | 10 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 116 | 21-6-2014 | 2 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Anaplasma phagocytophilum*, *Theileria/Babesia* catch-all, *Babesia* catch-all 1, *Babesia divergens*, *Babesia venatorum (sp EU 1)* and *Rickettsia helvetica.* |
| 117 | 21-6-2014 | 2 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | Negative for every pathogen |
| 123 | 22-6-2014 | 4 | I. Ricinus | Nymph | Song thrush | Voorboezem | positive *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva* |
| 124 | 22-6-2014 | 1 | I. Ricinus | Nymph | Song thrush | Voorboezem | positive *Borrelia garinii* |
| 125 | 22-6-2014 | 1 | I. Ricinus | Nymph | Song thrush | Voorboezem | positive Theileria/Babesia catch-all, Theileria catch-all, Theileria parva and *Borrelia garinii* |
| 131 | 24-6-2014 | 10 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 137 | 25-6-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | Negative for every pathogen |
| 138 | 25-6-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all and *Anaplasma phagocytophilum.* |
| 160 | 7-7-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | positive *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva* |
| 171 | 14-7-2014 | 2 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Ehrlichia canis*, *Theileria/Babesia* catch-all, *Theileria* catch-all, *Theileria parva* and *Borrelia garinii* |
| 185 | 21-7-2014 | 1 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Theileria/Babesia* catch-all, *Theileria* catch-all and *Theileria parva* |
| 199 | 10-8-2014 | 9 | I. Ricinus | Nymph | Dunnock | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Theileria* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica.* |
| 215 | 22-8-2014 | 1 | I. Ricinus | Nymph | Eurasian wren | Leemans Gemaal | Negative for every pathogen |
| 224 | 8-9-2014 | 2 | I. Ricinus | Nymph | Eurasian wren | Leemans Gemaal | positive *Babesia* catch-all 1. |
| 239 | 6-10-2014 | 1 | I. Ricinus | Nymph | Chaffinch | Leemans Gemaal | Negative for everything |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 260 | 27-10-2014 | 2 | I. Ricinus | Nymph | Redwing | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all and *Anaplasma phagocytophilum.* |
| 263 | 31-10-2014 | 1 | I. Ricinus | Nymph | Redwing | Leemans Gemaal | Negative for every pathogen |
| 279 | 9-11-2014 | 2 | I. Ricinus | Nymph | Redwing | Leemans Gemaal | Negative for every pathogen |
| 285 | 9-11-2014 | 1 | I. Ricinus | Nymph | Treecreeper | Leemans Gemaal | Negative for every pathogen |
| 4 | 4-1-2015 | 1 | I. Ricinus | Nymph | Redwing | Leemans Gemaal | Negative for every pathogen |
| 10 | 16-1-2015 | 2 | I. Ricinus | Nymph | Redwing | Leemans Gemaal | Negative for every pathogen |
| 11 | 16-1-2015 | 1 | I. Ricinus | Nymph | Redwing | Leemans Gemaal | Negative for every pathogen |
| 17 | 1-2-2015 | 1 | I. Ricinus | Nymph | Redwing | Leemans Gemaal | Negative for every pathogen |

**RLB 3**

| Ticknr | Detectiondate | Number | Tick Specie | Stage | Host | Location | RLB Results |
|---|---|---|---|---|---|---|---|
| 142 | 23-11-2013 | 1 | Ixodes ricinus | Nymph | Common blackbird | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all |
| 143 | 23-11-2013 | 2 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 145 | 23-11-2013 | 2 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 146 | 24-11-2013 | 1 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 147 | 24-11-2013 | 2 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 148 | 24-11-2013 | 1 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive *Theileria/Babesia* catch-all, *Babesia* catch-all 1 and *Babesia venatorum* (sp EU1). |
| 149 | 30-11-2013 | 1 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 150 | 30-11-2013 | 1 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 153 | 8-12-2013 | 1 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 155 | 8-12-2013 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 157 | 13-12-2013 | 2 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaa | positive *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica.* |
| 158 | 20-12-2013 | 3 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 21 | 6-3-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 41 | 24-3-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 44 | 5-4-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Theileria/Babesia* catch-all, *Babesia* catch-all 1 and *Babesia venatorum* (sp EU1). |
| 47 | 6-4-2014 | 3 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Theileria/Babesia* catch-all and *Theileria equi*. |
| 67 | 1-5-2014 | 2 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 75 | 30-5-2014 | 3 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 78 | 2-6-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 85 | 7-6-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 90 | 7-6-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 92 | 7-6-2014 | 2 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 93 | 7-6-2014 | 2 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 148 | 7-7-2014 | 4 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 161 | 7-7-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | Negative for every pathogen |
| 162 | 13-7-2014 | 3 | I. Ricinus | Nymph | Song thrush | Voorboezem | Negative for every pathogen |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 168 | 14-7-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | Negative for every pathogen |
| 169 | 14-7-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | Negative for every pathogen |
| 174 | 15-7-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | Negative for every pathogen |
| 180 | 16-7-2014 | 5 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 188 | 23-7-2014 | 1 | I. Ricinus | Nymph | Eurasian blackcap | Leemans Gemaal | Negative for every pathogen |
| 191 | 10-8-2014 | 2 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all |
| 197 | 10-8-2014 | 2 | I. Ricinus | Nymp | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 200 | 10-8-2014 | 3 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 203 | 12-8-2014 | 2 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 232 | 22-9-2014 | 2 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 236 | 27-9-2014 | 8 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |
| 238 | 5-10-2014 | 1 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | positive Theileria/Babesia catch-all. |
| 241 | 10-10-2014 | 1 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica.* |
| 242 | 10-10-2014 | 3 | I. Ricinus | Nymph | Song thrush | Leemans Gemaal | Negative for every pathogen |

**RLB 4**

| Ticknr | Detectiondate | Number | Tick Species | Stage | Host | Location | RLB Results |
|---|---|---|---|---|---|---|---|
| 1 | 26-1-2014 | 1 | Ixodes Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 2 | 26-1-2014 | 1 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 3 | 26-1-2014 | 1 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 4 | 26-1-2014 | 1 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all and *Ehrlichia ruminantium* |
| 5 | 22-2-2014 | 2 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 7 | 23-2-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 8 | 22-2-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 9 | 22-2-2014 | 3 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 10 | 26-2-2014 | 10 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Theileria/Babesia* catch-all, *Babesia* catch-all 1 and *Babesia venatorum* (sp EU 1) |
| 11 | 1-3-2014 | 7 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive *Theileria/Babesia* catch-all, *Babesia* catch-all 1 and *Babesia venatorum* (sp EU 1) |
| 12 | 1-3-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 13 | 1-3-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 15 | 1-3-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica* |
| 16 | 3-3-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 17 | 3-3-2014 | 2 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 18 | 6-3-2014 | 2 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 19 | 6-3-2014 | 2 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 101 | 16-6-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 102 | 16-6-2014 | 3 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 106 | 16-6-2014 | 2 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Theileria/Babesia* catch-all, and *Rickettsia helvetica* |

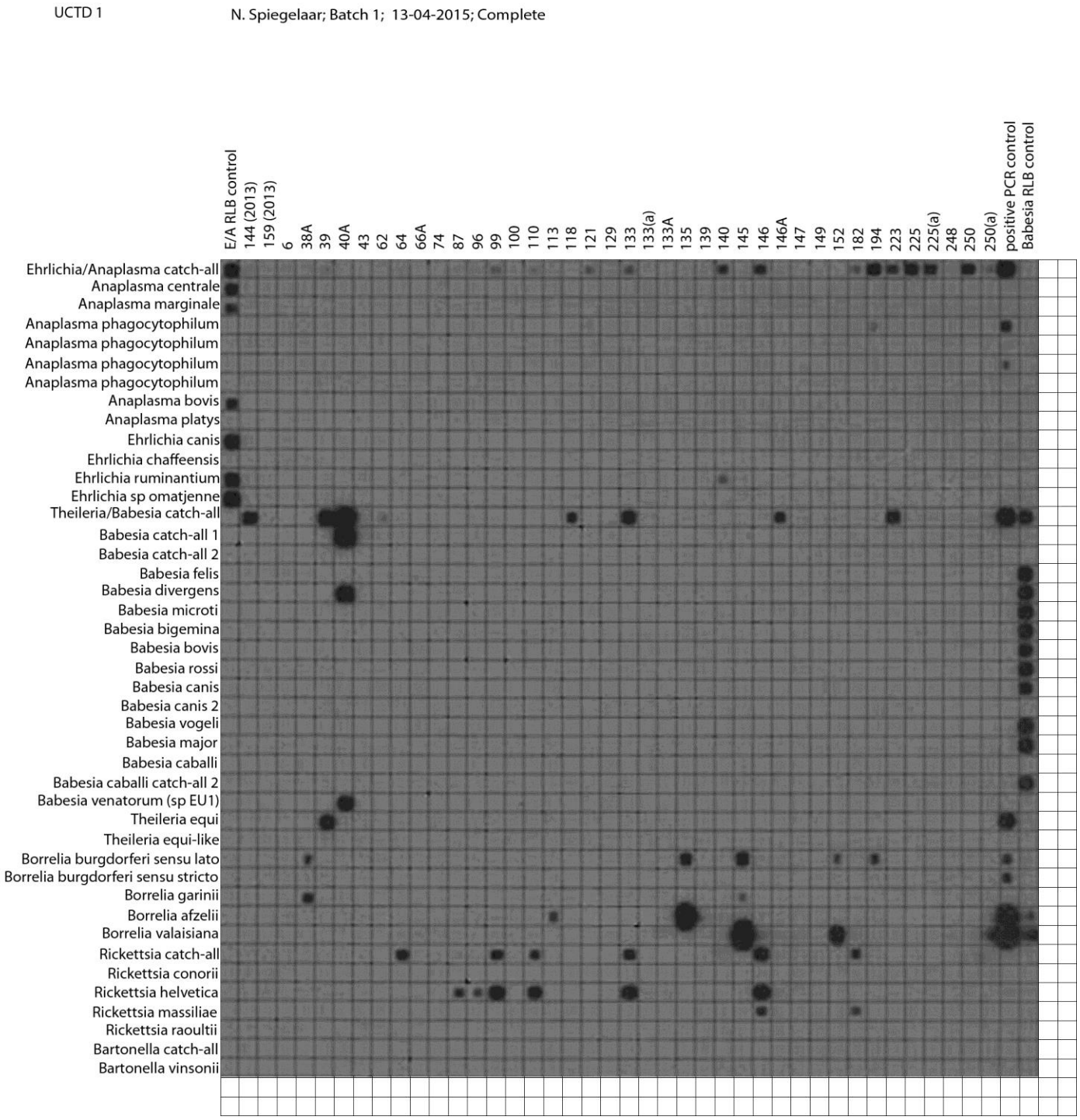
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 108 | 21-6-2014 | 4 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all |
| 111 | 21-6-2014 | 6 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica* |
| 112 | 21-6-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 128 | 24-6-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 132 | 24-6-2014 | 4 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 134 | 25-6-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Babesia* catch-all 1 |
| 142 | 28-6-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Rickettsia helvetica* |
| 151 | 7-7-2014 | 2 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 155 | 7-7-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Anaplasma phagocytophilum, Rickettsia* catch-all and *Rickettsia helvetica* |
| 156 | 7-7-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 157 | 7-7-2014 | 2 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 176 | 15-7-2014 | 2 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica* |
| 181 | 17-7-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all |
| 182a | 17-7-2014 | 3 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all |
| 208 | 14-8-2014 | 3 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 209 | 14-8-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 212 | 22-8-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | Negative for every pathogen |
| 222 | 8-9-2014 | 3 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all |
| 227 | 13-9-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Anaplasma phagocytophilum* |
| 247 | 10-10-2014 | 1 | I. Ricinus | Nymph | European robin | Leemans Gemaal | positive *Ehrlichia/Anaplasma* catch-all, *Rickettsia catch-all* and *Rickettsia helvetica* |

**RLB 5**

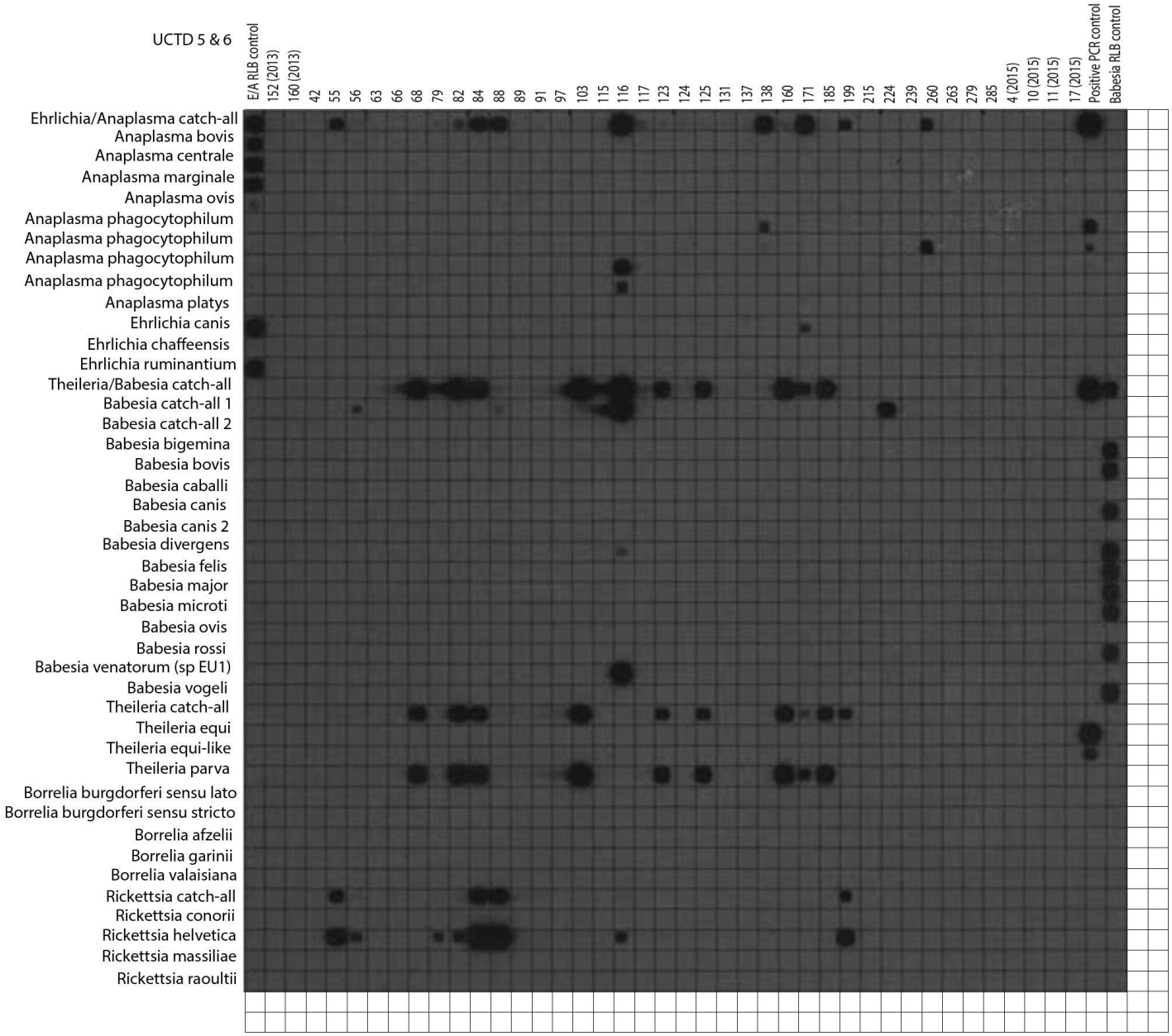
| Ticknr | Detectiondate | Number | Tick Specie | Stage | Host | Location | RLB Results |
|---|---|---|---|---|---|---|---|
| 20 | 6-3-2014 | 1 | Ixodes Ricinus | Nymph | Great tit | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all and *Rickettsia helvetica.* |
| 22 | 6-3-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all, *Neoehrlichia mikurensis, Rickettsia* catch-all and *Rickettsia helvetica.* |
| 24 | 6-3-2014 | 5 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica.* |
| 35 | 17-3-2014 | 10 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all, *Neoehrlichia mikurensis*, *Borrelia burgdorferi* sensu lato and *Borrelia garinii.* |
| 37 | 22-3-2014 | 3 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch-all and *Rickettsia helvetica.* |
| 38 | 23-3-2014 | 2 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all, *Theileria* catch-all, *Borrelia garinii, Borrelia valaisiana, Rickettsia* catch-all and *Rickettsia helvetica.* |
| 46 | 6-4-2014 | 1 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive for *Theileria* catch-all and *Rickettsia helvetica.* |
| 48 | 6-4-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 49 | 6-4-2014 | 2 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 50 | 7-4-2014 | 2 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 51 | 12-4-2014 | 6 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 52 | 12-4-2014 | 2 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all, *Neoehrlichia mikurensis, Borrelia valaisiana, Rickettsia* catch-all and *Rickettsia helvetica.* |
| 53 | 12-4-2014 | 1 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 57 | 23-4-2014 | 1 | I. Ricinus | Nymph | Common blackbird | Voorboezem | Negative for every pathogen |
| 61 | 25-4-2014 | 10 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive for *Borrelia burgdorferi* sensu lato and *Borrelia valaisiana.* |
| 65 | 1-5-2014 | 10 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | Negative for every pathogen |
| 71 | 16-5-2014 | 1 | I. Ricinus | Nymph | Great tit | Voorboezem | Negative for every pathogen |
| 77 | 1-6-2014 | 10 | I. Ricinus | Nymph | Common blackbird | Voorboezem | positive for *Borrelia valaisiana.* |
| 80a | 2-6-2014 | 10 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all, *Borrelia burgdorferi* sensu lato, *Borrelia garinii, Borrelia valaisiana* and *Rickettsia helvetica.* |
| 95 | 14-6-2014 | 8 | I. Ricinus | Nymph | Common blackbird | Voorboezem | positive for *Borrelia burgdorferi* sensu lato and *Borrelia valaisiana.* |
| 105 | 16-6-2014 | 10 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all, *Borrelia burgdorferi* sensu lato, *Borrelia garinii* and *Rickettsia helvetica.* |
| 114 | 21-6-2014 | 10 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive for *Theileria* catch-all, *Borrelia burgdorferi* sensu lato, *Borrelia garinii and Borrelia valaisiana*. |
| 122 | 21-6-2014 | 10 | I. Ricinus | Nymph | Common blackbird | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all, *Borrelia burgdorferi* sensu lato, *Borrelia garinii, Borrelia valaisiana* and *Rickettsia helvetica*. |
| 140a | 28-6-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 175 | 15-7-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 218 | 28-8-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | positive for *Ehrlichia/Anaplasma* catch-all, *Rickettsia* catch/all and *Rickettsia helvetica*. |
| 230 | 21-9-2014 | 2 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 163 | 13-7-2014 | 2 | I. Ricinus | Nymph | Common blackbird | Voorboezem | Negative for every pathogen |
| 234 | 27-9-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |
| 251 | 13-10-2014 | 1 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | positive for *Theileria/Babesia* catch-all and *Theileria equi*. |
| 253 | 13-10-2014 | 2 | I. Ricinus | Nymph | Great tit | Leemans Gemaal | Negative for every pathogen |

## Appendix E: RLB results

### Batch 1: Complete



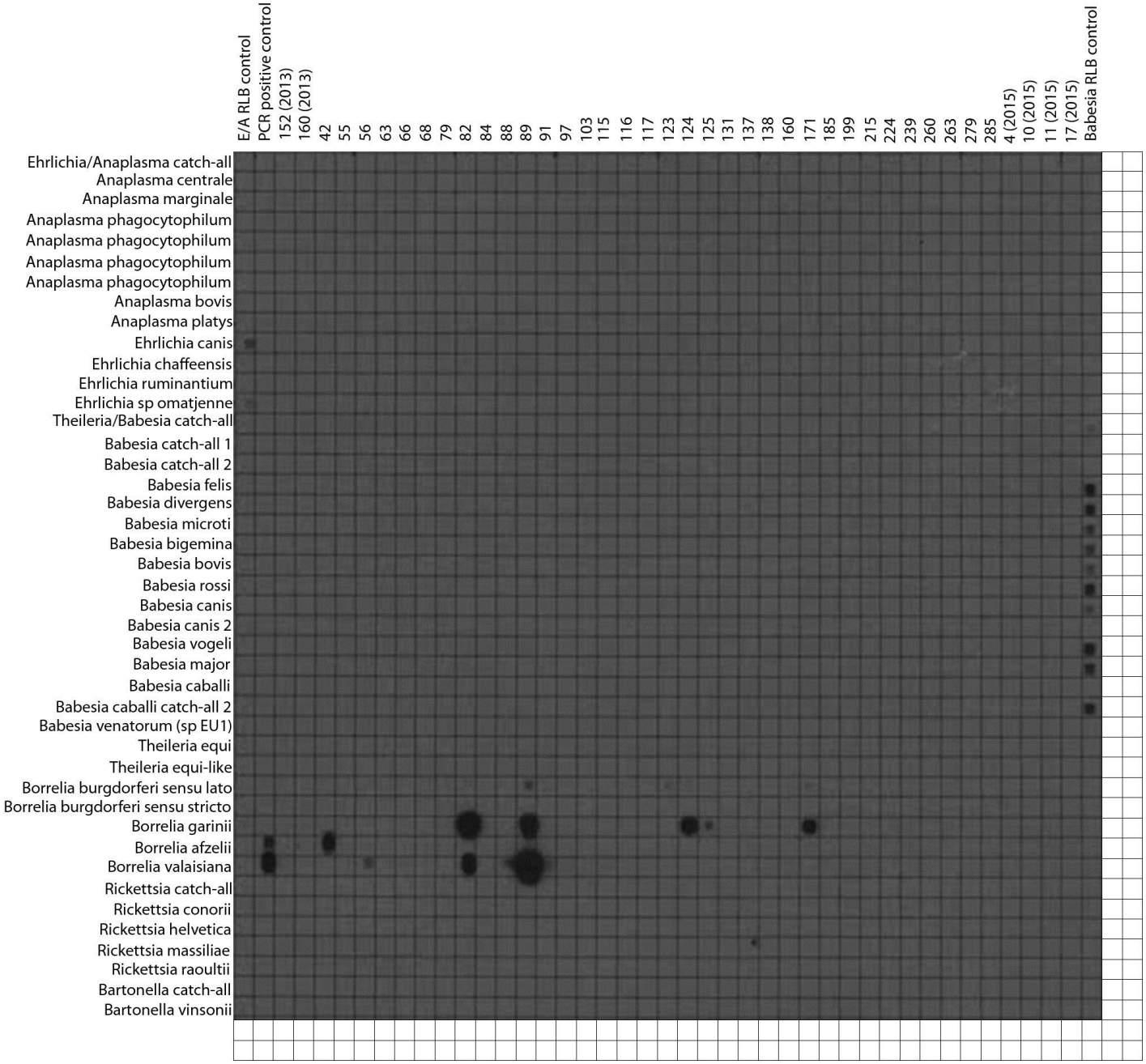
## Batch 2: *Anaplasma/Theileria*



## Batch 2: *Borrelia*

UCTD 1

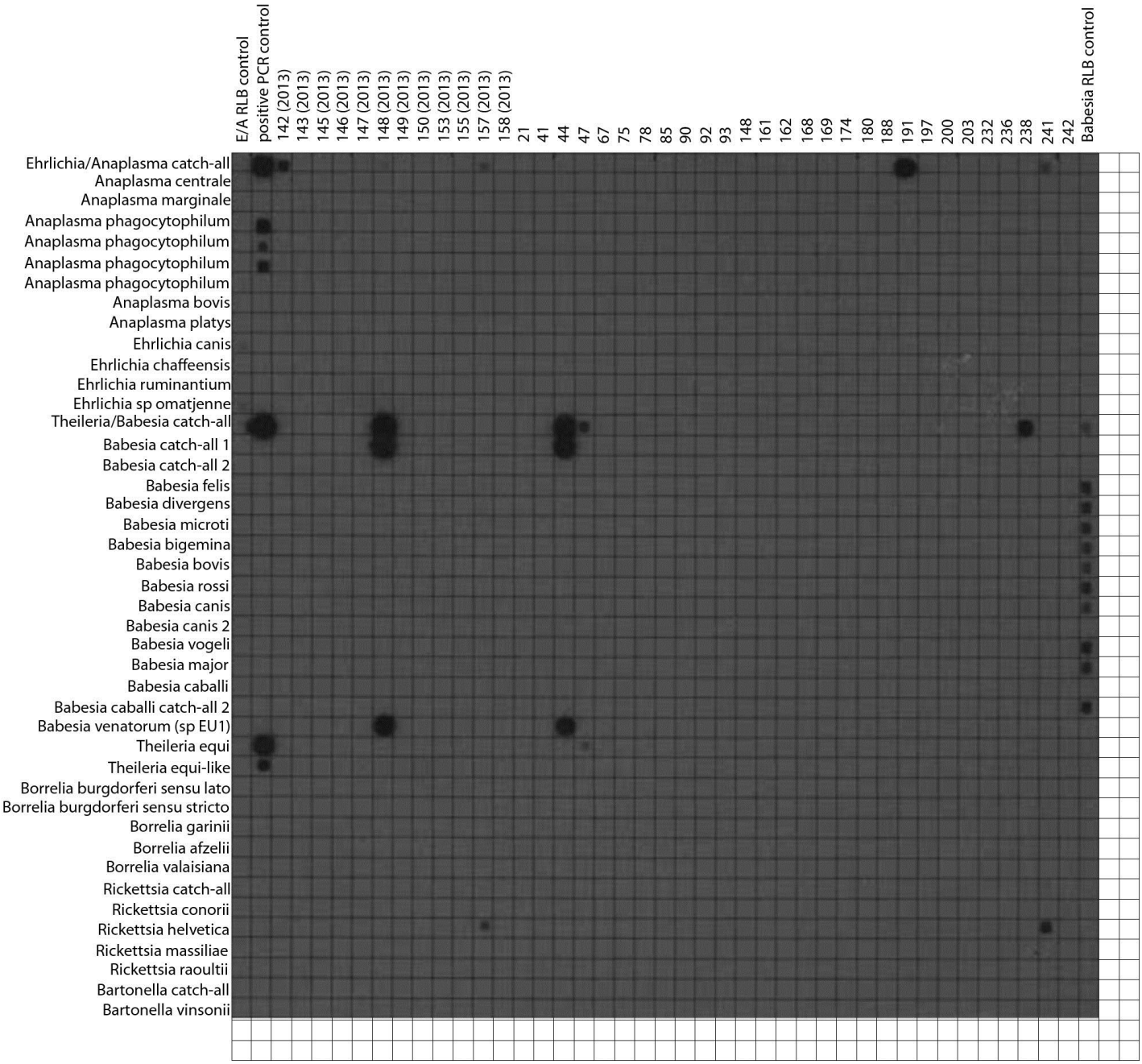
N. Spiegelaar; Batch 2; 29-04-2015; Borrelia



## Batch 3: *Anaplasma/Theileria*

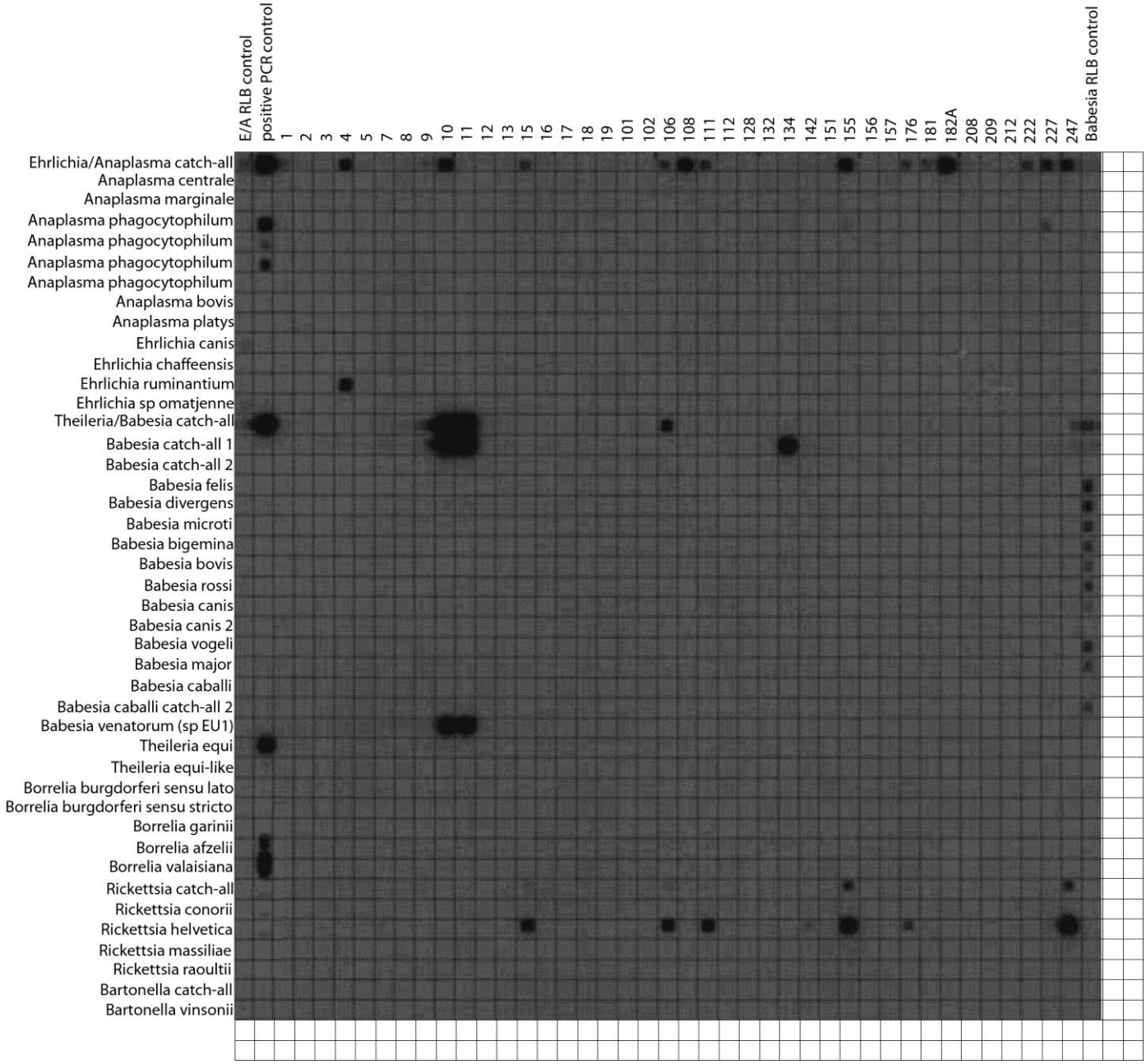
UCTD 1

N. Spiegelaar; Batch 3; 22-04-2015; Anaplasma/Theileria



## Batch 4: Complete

UCTD 1 N. Spiegelaar; Batch 4; 23-04-2015; Complete



## **Batch 5**: **Complete**